DESCRIPTION

Therapeutic agent for cerebral neurodegenerative diseases via PPARδ agonist

Technical Field

The present invention relates to the use of a compound with a PPARδ agonist activity as a therapeutic agent for cerebral neurodegenerative diseases. Additionally, the invention relates to a method for therapeutically treating cerebral neurodegenerative diseases which comprises administering a pharmaceutical agent containing the compound as an active ingredient.

Background of the Invention

Various central diseases such as cerebral infarction, Parkinson's disease, Alzheimer's disease and Huntington's disease are all caused by the degeneration of nerve cells and trigger various severe disorders.

As well known, cerebral tissues are damaged by for example the occlusion of cerebral blood tubes, resulting in no blood flow (cerebral infarction) and the rupture of cerebral blood tubes, leading to bleeding (cerebral hemorrhage), so that blood flow enough for cerebral nerve cells to be viable cannot be securely retained. Accordingly, brain falls into necrosis in a very short time. The death of

nerve cells following such ischemia starts from the necrosis of nerve cells in the core region of an infarct lesion directly exposed to an ischemic state and spreads gradually to the penumbra, so that damages spread to a great number of

5   nerve cells. Among them, the necrosis of the core region is caused irreversibly in such a very short time that it is considered that substantial treatment thereof is impossible. Because the death of nerve cells in the penumbra gradually progresses under the influence of the necrosis of adjacent

10 nerve cells, however, it is expected that the death thereof may possibly be suppressed in a reversible manner at some extent at the initial stage. Therefore, various attempts have been made in such expectation to develop various therapeutic agents. Currently, however, the molecular

15 mechanism of the degeneration of nerve cells in such ischemic state has not yet been sufficiently elucidated. No pharmaceutical agent with a clearly verified therapeutic effect on the suppression of the death of nerve cells on clinical site has been found.

20   Meanwhile, Parkinson's disease (PD) is a central disease caused by gradual degeneration of nigrostriatal dopaminergic neurons over a long time, which causes symptoms such as tremor, muscular rigor and akinesia. The disease is a disease at a high incident rate per population but the

25 detailed etiology causing the onset has not yet been elucidated. It is supposed that genetic factors, endogenous

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and exogenous toxins, oxidative stress and the like may subtly give an influence on the exacerbation of the disordered conditions. In this disease, only nigrostriatal dopaminergic neurons are selectively degenerated among

5 numerous nerve cells. Not any pharmaceutical agent with a clinically clearly certified potency to significantly suppress the neurodegeneration to substantially suppress the progress of Parkinson's disease itself has existed yet.

Therefore, the development of a pharmaceutical agent

10 suppressing neurodegeneration or a pharmaceutical agent promoting the regeneration of damaged nerves has been strongly desired so as to essentially treat severe diseases such as cerebral infarction and Parkinson's disease. At a first step for finding a clinically effective pharmaceutical

15 agent, generally, an approach is now under way for finding a compound suppressing the death of nerve cells for example culture cells of the nerve system by screening using various target protein molecules suspected to be involved in the course of neurodegeneration and then certifying the effect of

20 such compound in such central diseases-mimetic models of animals such as mouse and rat. It is considered that the discovery of a new target protein may be an important step for the discovery of a new therapeutic agent. Up to now, a great number of genetic products suspected to be responsible

25 for the course of neurodegeneration have been found by

analyses using experimental model animals mainly having mimetic cerebral infarction state.

Herein, peroxisome proliferator-activated receptors (PPAR) found as proteins mediating the function of increasing cellular small organelle peroxisome involved in fat degradation are members of the superfamily of transcription factors and nuclear receptors employing glucocorticoid, estrogen, progesterone, thyroid hormone and fat-soluble vitamins as the ligands. A great number of research works so far have indicated that PPAR has very important functions in controlling many genetic expressions and is involved in numerous diseases. Accordingly, the relation of PPAR with the death of nerve cells has been drawing attention.

It has been elucidated until now that human PPAR includes at least three sub-types of α, γ and δ. Each of the PPAR sub-types forms a hetero-dimer with RXR, using 9-cis retinoic acid as the ligand and is responsible for the regulation of the expression of diverse genes with the PPAR responsible element (PPRE: 5'-AGGTCA-X-AGGTCA-3') in the promoter regions. During the binding of such ligands to the PPAR-RXR hetero-dimer, it is generally considered that the dissociation of the co-repressor and the association to the co-activator occur, leading to the exertion of the transcription activation potency.

PPARα is highly expressed in tissues with high levels of fatty acid utilization, such as liver, kidney, heart and

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gastrointestinal tract and plays a role of regulating the metabolism of fatty acids, particularly the oxidation of fatty acids. Additionally, it is anticipated from research works on PPARα knockout mice that PPARα is closely involved

5   in the onset of insulin resistance with highly fatty diets.

It is known that PPARγ has an important role in differentiation into fat cells. It is known for example that differentiation into fat cells is induced by causing PPARγ expression in fibroblast cells and subsequent treatment with

10   a thiazolidine derivative (thiazolidinedione: TZD) as a potent synthetic PPARγ ligand. Further, the thiazolidine derivative is an agent for ameliorating the insulin resistance of type 2 diabetes accompanied by obesity. PPARγ induces differentiation into fat cells when exposed to a

15   relatively high concentration of the ligands, such as in the case of administration of the thiazolidine derivative, leading to the increase of small-type fat cells and the apoptosis of enlarged fat cells. Consequently, PPARγ works for the activation of insulin sensitivity. When exposed to a

20   relatively low concentration of the ligands such as in the case of a load of highly fatty diet (HF) in the absence of the thiazolidine derivative, it is considered that PPARγ works for the enlargement of fat cells, fat accumulation and insulin resistance.

25   Alternatively, PPARδ is expressed in diverse tissues such as brain, liver, kidney, spleen, fat, skeletal muscle,

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digestive tube, skin and placenta. It is reported that PPARδ has functions in fat cell differentiation, cerebral functions and epidermal differentiation. About 90 % of PPARδ knockout mice are dead at viviparity. Even when such mice are born,

5   poor growth is observed throughout the viviparity to post-delivery, compared with the wild type. Furthermore, it is observed that their brains are small in proportion to the body sizes and myelin formation in the corpus callosum is abnormal. Additionally, epidermal hypertrophy is

10   significantly enhanced in the knockout mice. As described above, it is suggested that PPARδ is closely involved in development, lipid metabolism, cerebral myelin formation, and epidermal cell growth. Still additionally, some reports tell about the activation of the cholesterol reverse transport

15   system, the improvement of the composition ratio of lipoproteins and the decrease of neutral fat, with PPARδ selective agonists. PPARδ is a sub-type abundantly expressed in brain but almost no physiological role of PPARδ in brain has been elucidated.

20   The relation between the role of each of these PPAR sub-types and the neurodegenerative diseases has not yet been fully elucidated. However, so far, reports suggest that, regarding PPARγ which has been relatively increasingly elucidated, compounds with PPARγ agonist activities have some

25   therapeutic effects on cerebral infarction and Parkinson's disease in animal models. For example, S. Sundararajan et

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al. report that PPARγ agonists are effective in rat models of cerebral infarction (S. Sundararajan, W.D. Lust, D.M.D. Landis and G.E. Landreth, Soc. Neurosci. Abstr. <u>26</u> (2000), p1808. PPAR gamma agonists reduce ischemic injury and

5   immunoreactivity against inflammatory markers in rats.)

Further, S. Uryu et al. report that troglitazone suppresses the cellular death of cerebellar granule neurons and tells about an expectation of its use as a suppressing agent for the death of nerve cells. However, it is not

10 clearly shown that PPARγ agonists have a general relation with the suppression of the death of nerve cells. (Shigeko Uryu, Jun Harada, Marie Hisamoto and Tomiichiro Oda. Brain Research 924 (2002) p229-236. Troglitazone inhibits both post-glutamate neurotoxicity and low-potassium-induced

15 apoptosis in cerebellar granule neurons.)

Additionally, T. Breidert et al. report that one PPARγ agonist, pioglitazone, protectively works against the neurodegeneration of a mouse model of MPTP-induced Parkinson's disease (T. Breidert, J. Callebert, M.T. Heneka,

20 G. Landreth, J.M. Launay and E.C. Hirsch. Journal of Neurochemistry 82 (2002) p615-624. Protective action of the peroxisome proliferator-activated receptor-gamma agonist pioglitazone in a mouse model of Parkinson's disease.) This may be ascribed to the anti-inflammatory effect of the PPARγ

25 agonist.

WO0249626A2 discloses a therapeutic method for neurodegenerative diseases such as cerebral infarction and Parkinson's disease with PPARγ agonist.

Additionally, WO0213812A1 discloses a therapeutic method for neurodegenerative diseases and inflammatory diseases with PPARγ agonist. It specifies cerebral infarction, Parkinson's disease and Alzheimer's disease as the neurodegenerative diseases and also discloses that a PPARγ/PPARδ dual agonist has the same therapeutic effect. The specification however never includes any reference to the effect of the PPARδ agonist alone. The specification simply describes that the effect of the PPARγ agonist with low sub-type selectivity is due to the effect of the PPARγ/PPARδ dual agonist but never clearly verifies or discriminates the action of the PPARδ agonist from the action of the PPARγ agonist.

Herein, the possibility of a number of severe adverse actions of thiazolidinedione-series compounds such as rosiglitazone and pioglitazone as typical PPARγ agonists has been suggested. Among them, an adverse action of water retention is found at clinical tests of the pharmaceutical agents as therapeutic agents of type 2 diabetes and at various animal experiments (Sood V, Colleran K, Burge MR. Diabetes Technol Ther 2 (2000) p429-440. Thiazolidinediones: a comparative review of approved uses.) The enhancement of water retention often leads to cerebral edema and draw

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concerns about the exacerbating action of cerebral infarction. Therefore, the development of these PPARγ agonists as therapeutic agents of cerebral infarction may involve essential difficulty.

5   On the other hand, currently, no report tells that PPARδ has a direct relation with the onset of neurodegenerative diseases such as cerebral infarction and Parkinson's disease. No report has been known, telling a relation with edema. Nonetheless, some reports exist

10   suggesting that PPARδ has a relation with apoptosis and inflammatory cellular death.

For example, T. Hatae et al. report apoptosis induction by the introduction of an expression vector of the prostacyclin synthase gene in human fetus kidney-derived 293

15   cell and activation of PPARδ (Toshihisa Hatae, Masayuki Wada, Chieko Yokoyama, Manabu Shimonishi and Tadashi Tanabe. Prostacyclin-dependent Apoptosis Mediated by PPARδ. The Journal of Biological Chemistry 276 (2001) pp.46260-46267).

Furthermore, WO0107066A1 describes that PPARδ

20   inhibitors when administered inhibit the course of forming foam cell from macrophage and can therefore therapeutically treat various vascular diseases. The specification exemplifies cerebral stroke and Alzheimer's disease as various vascular diseases to be possibly treated. However,

25   the specification simply discloses that PPARδ expressed in macrophage induces inflammatory reactions so that PPARδ is a

factor exacerbating these diseases. In contrast, the role of PPARδ expressed in cells of the central nervous system or the possibility of PPARδ agonist as a therapeutic agent is absolutely never described.

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Disclosure of the Invention

Research works so far suggest that some of compounds with PPARγ agonist activities have a function to suppress neurodegenerative progress in cerebral infarction and the

10   like. Since individual compounds with PPARγ agonist activities have more or less agonist activities simultaneously for PPARα and PPARδ in many cases, however, the relation between the agonist activity for each of the PPAR sub-types and the therapeutic effect on

15   neurodegenerative diseases has not yet been fully elucidated. No report tells that a PPARγ agonist itself directly suppresses the death of neuron cells. Rather, an effect of indirectly suppressing nerve cell death via the essential anti-inflammatory action of such PPARγ agonist is highly

20   possible. This suggests that the therapeutic effect of PPARγ agonist is limited only to the therapy of the inflammatory stage of neurodegenerative diseases involving inflammation. Additionally, it has been known so far that many of PPARγ agonists cause severe adverse actions such as water

25   retention. Because cerebral edema is highly possibly exacerbated during the therapy of the cerebral nerve system,

the possibility makes the application to clinical practice very tough. Therefore, it is highly demanded to select a compound capable of directly suppressing the death of neuron cells and effective for therapeutic treatment of various,

5   diverse nerve diseases without any adverse actions such as water retention and to provide a great method applicable to clinical practice. It is an object of the present invention to overcome such problems. Present research works made by the inventors have first elucidated that agonists specific to

10 PPARδ are singly effective for neurodegenerative diseases such as cerebral infarction and Parkinson's disease. Further, the research works have elucidated that PPARδ agonist directly interacts with neuron cell to suppress the death of the cell. These results indicate that PPARδ agonist

15 may be effective for more diverse neurodegenerative diseases, compared with PPARγ agonist.

During the course of research works about investigations for culture cells derived from the nerve system and the relation between the actions of various nerve

20 toxins and the death of nerve cells, the inventors have first found a fact that PPARδ agonist significantly suppresses the death of nerve cells due to thapsigargin, MPP^+, staurosporine and the like. Therefore, the inventors have assessed the efficacy of these PPARδ agonists in model animals of cerebral

25 infarction. The inventors have verified that these PPARδ agonists actually have an action of suppressing the death of

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nerve cells at an ischemic state following cerebral infarction. Additionally, the inventors have verified that the PPARδ agonists resume the MPTP-induced decrease of intracerebral dopamine content in a model animal of MPTP-induced Parkinson's disease. Additionally, the inventors have found that compounds with PPARδ agonist activities are useful for the therapeutic treatment of neurodegenerative diseases such as cerebral infarction and Parkinson's disease and that an efficacious therapeutic agent therefor can be selected among such compounds.

Specifically, the invention relates to a therapeutic method for Alzheimer's disease, Parkinson's disease, cerebral infarction, head injuries, cerebral hemorrhage, spinal injuries, multiple sclerosis, amyotrophic lateral sclerosis, Huntington's disease, diabetic or drug-induced peripheral nerve disorders or retinal nerve disorders, which comprises administering a therapeutic agent for neurodegenerative diseases, the therapeutic agent containing a PPARδ agonist as an active ingredient.

Additionally, the invention relates to a therapeutic agent for Alzheimer's disease, Parkinson's disease, cerebral infarction, head injuries, cerebral hemorrhage, spinal injuries, multiple sclerosis, amyotrophic lateral sclerosis, Huntington's disease, diabetic or drug-induced peripheral nerve disorders or retinal nerve disorders, the therapeutic agent containing a PPARδ agonist as an active ingredient.

In other words, the invention relates to those described below.

(1)   A therapeutic agent for Alzheimer's disease, Parkinson's disease, cerebral infarction, head injuries, cerebral hemorrhage, spinal injuries, multiple sclerosis, amyotrophic lateral sclerosis, Huntington's disease, diabetic or drug-induced peripheral nerve disorders or retinal nerve disorders, which comprises a PPARδ agonist as an active ingredient.

(2)   A therapeutic method for Alzheimer's disease, Parkinson's disease, cerebral infarction, head injuries, cerebral hemorrhage, spinal injuries, multiple sclerosis, amyotrophic lateral sclerosis, Huntington's disease, diabetic or drug-induced peripheral nerve disorders or retinal nerve disorders, which comprises administering a pharmaceutical agent comprising a PPARδ agonist as an active ingredient.

(3)   A therapeutic agent for cerebral infarction, which comprises a PPARδ agonist as an active ingredient.

(4)   A therapeutic agent for Parkinson's disease, which comprises a PPARδ agonist as an active ingredient.

(5)   A therapeutic method for cerebral infarction, which comprises administering a pharmaceutical agent comprising a PPARδ agonist as an active ingredient.

(6)   A therapeutic method for Parkinson's disease, which comprises administering a pharmaceutical agent comprising a PPARδ agonist as an active ingredient.

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(7)   The therapeutic agent described above in (1),
(3) or (4), wherein the PPARδ agonist is a PPARδ agonist
specifically re-selected using the activity of suppressing
cellular death as the marker.

5   (8)   The therapeutic method described above in (2),
(5) or (6), wherein the PPARδ agonist is a PPARδ agonist
specifically re-selected using the activity of suppressing
cellular death as the marker.

(9)   The therapeutic agent described above in (1),
10   (3) or (4), where the PPARδ agonist is L-165041 or GW501516.

(10)   The therapeutic method described above in (2),
(5) or (6), wherein the PPARδ agonist is L-165041 or
GW501516.

(11)   Use of a PPARδ agonist for the manufacture of a
15   therapeutic agent for Alzheimer's disease, Parkinson's
disease, cerebral infarction, head injuries, cerebral
hemorrhage, spinal injuries, multiple sclerosis, amyotrophic
lateral sclerosis, Huntington's disease, diabetic or drug-
induced peripheral nerve disorders or retinal nerve
20   disorders.

(12)   Use of a PPARδ agonist for the manufacture of a
therapeutic agent for cerebral infarction.

(13)   Use of a PPARδ agonist for the manufacture of a
therapeutic agent for Parkinson's disease.

25   (14)   The use described above in any of (11) through
(13), wherein the PPARδ agonist is a PPARδ agonist

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specifically re-selected using the activity of suppressing cellular death as the marker.

(15)   The use described above in any of (11) through (13), wherein the PPARδ agonist is L-165041 or GW501516.

(16)   An agent of suppressing the death of central nerve cell, which comprises a PPARδ agonist as an active ingredient.

(17)   The agent of suppressing the death of central nerve cell as described above in (16), wherein the PPARδ agonist is L-165041 or GWW501516.

The invention is now described in detail hereinbelow.

The invention relates to a therapeutic agent for Alzheimer's disease, Parkinson's disease, cerebral infarction, head injuries, cerebral hemorrhage, spinal injuries, multiple sclerosis, amyotrophic lateral sclerosis, Huntington's disease, diabetic or drug-induced peripheral nerve disorders or retinal nerve disorders, the therapeutic agent containing a PPARδ agonist as an active ingredient, as well as a therapeutic method thereof.

The PPARδ agonist in accordance with the invention is also referred to as PPARδ-like functioning agent or PPARδ-like acting agent and is a low molecular compound with various physiological actions by specifically binding to the PPARδ protein as a nuclear receptor to induce the modification of the structure, promoting the binding of the

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PPARδ-RXR complex to PPRE (peroxisome proliferator response element) and also promoting the expression of various genes with the PPRE sequence in the promoter regions.

5   Known compounds with the typical activities of the PPARδ agonist include for example L-165041 and GW501516.

It is reported that L-165041 (4-[3-[2-propyl-3-hydroxy-4-acetyl]phenoxy]propyloxyphenoxy acetic acid) has an activity binding to both PPARδ and PPARγ but its affinity to

10   PPARγ (Ki 730 nM) is much weaker than its affinity to PPARδ (Ki 6 nM) (Mark D. Leibowitz et al., Activation of PPARδ alters lipid metabolism in db/db mice. FEBS Letters 473(2000) 333-336).

It is reported that GW501516 is a compound with a high

15   affinity to PPARδ (Ki= 1.1 ± 0.1 nM) and exerts a high expression induction activity when the agonist activity is assayed using the GAL4-resposive reporter gene (EC$_{50}$ = 1.2 ± 0.1 nM) and that its effect involves a selectivity 1000-fold or more to PPARδ, compared with the selectivity to other PPAR

20   sub-types (William R. Oliver, Jr. et al., A selective peroxisome proliferator-activated receptor δ agonist promotes reverse cholesterol transport. Proc. Natl. Acad. Sci. USA 98(2001) 5306-5311.)

The aforementioned reference information is supported

25   by the preliminary experimental results made by the inventors. Specifically, the inventors verified about the

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selectivity of L-165041 and GW501516 to human and murine-derived individual PPAR sub-types on the basis of the assessment of the action of fusion proteins of GAL4-individual PPAR sub-types to activate transcription in the GAL4-responsive reporter gene that both the compounds had particularly high selectivity to human and murine PPARδ. It was also verified then that GW501516 had a higher selectivity and a higher expression induction activity than L-165041 (see Reference Example 1, Table 7).

Other than L-165016 and GW501516, numerous compounds have already been reported in WO2002100351, WO0200250048, WO0179197, WO0246154, WO0214291 and Japanese Patent Application No. 2001-354671 as existing agonists with relatively high activity to the subtype PPARδ. Additionally, Brown PJ et al. report compounds for example GW2433 (Brown PJ, Smith-Oliver TA, Charifson PS, Tomkinson NC, Fivush AM, Sterrnbach DD, Wade LE, Orband-Miller L, Parks DJ, Blanchard SG, Kliewer SA, Lehmann JM and Willson TM., Chem. Biol. (1997), p909-918. Identification of peroxisome proliferator-activated receptor ligands from a biased chemical library).

A person with ordinary experimental skills in the art can readily reselect a compound with a neuroprotective action according to the following selective method using culture cells, among these compounds.

The selective method of PPARδ agonist includes for example the following methods. But these methods are only illustrated. Thus, the selective method is not specifically limited to these methods. A person with ordinary skills in the art can readily optimize the details of these methods to develop and carry out the resulting method.

The reporter gene assay method for selecting PPAR agonists is known. For example, one hybrid reporter gene assay is reported, which employs the Gal4 transcription system of yeast and the Gal4-PPARδ fusion protein. (Lehman JM, More LB, Smith Oliver TA, Wilkinson WO, Willson TM, & Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). J. Biol. Chem. 270 (1995) 12953-12956.) The action of PPARδ to drive the yeast transcription system can be evaluated by the method.

As shown in the following references, PPAR agonists can be selected by reproducing the biological transcription system using a reporter assay system utilizing PPRE as the recognition response sequence of PPARδ and the full-length PPARδ.

(Dreyer C, Krey G, Keller H, Givel F, Helftenbein G, & Wahli W. Control of the peroxisomal β-oxidation pathway by a novel family of nuclear hormone receptors. Cell 68 (1992) 879-887.)

(Kliever SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ, Umesono K, & Evans RM. Differential expression and activation of a family of murine peroxisome activated-activated receptors. Proc. Natl. Acad. Sci., USA

5   91 (1994) 7355-7359.)

(Devchand PR, keller H, Peters JM, Vazquez M, Gonzalez FJ, & Wahli W. The PPAR α-leukotriene B4 pathway to inflammation control. Nature 384 (1996) 39-43.)

(Forman BM, Chen J, & Evans RM. Hypolipidemic drugs,

10   polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors a and d. Proc. Natl. Acad. Sci., USA 94 (1997) 4312-4317.)

(Basu Modak S, Braissant O, Escher P, Desvergne B, Honegger P, & Wahli W. Peroxisome proliferator-activated

15   receptor β regulates acyl-CoA synthetase 2 in reaggregated rat brain cell cultures. J. Biol. Chem. 274 (1999) 35881-35888.)

(He TC, Chan TA, Vogelstein B, & Kinzler KW. PPARδ is an APC-regulated target of nonsteroidal anti-inflammatory

20   drugs. Cell 99 (1999) 353-345.)

For constructing a reporter assay system, generally, a transcription regulation region conjugated with a promoter of a known sequence which can express constitutively the PPAR

25   responsible element (PPRE: 5'-AGGTCA-X-AGGTCA-3') in the promoter region is constructed. Then, a DNA construct

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artificially conjugated with a reporter gene is conjugated downstream the transcription regulation region. Additionally, an expression vector with the PPAR gene conjugated with a known gene promoter is constructed. These DNA constructs are introduced in appropriate culture cells (for example, CV-1 cell). Among various compounds then added, a compound capable of significantly increasing the expression level of the reporter gene is selected. As such known gene promoters, for example, promoters of SV40 virus initial gene and CMV IE gene may be used. However, the known gene promoters are not limited to them. A transcription regulation region with a general expression activity may be used satisfactorily. Such DNA construct can readily be constructed by a person with ordinary experimental skills in the art.

As to a sequence site essential for the transcription regulation of PPARδ and the like, an artificial DNA construct with plural such essential sequences in tandem repeat can be prepared by routine recombinant DNA experimental procedures. By using such artificial construct in replace of the natural sequence, the transcription-inducing activity of the transcription regulation region may sometimes be enhanced.

So as to accurately assay the PPARδ agonist activity, the method described above can be further improved to more accurately imitate the biological state. It is indicated that in biological organisms, PPARδ forms a heterodimer with

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RXR and further interacts with a co-activator of PPARδ, to exert its transcription activity. Therefore, the action of a compound with a PPARδ agonist activity to drive the PPARδ transcription system in a state more closely imitating the

5   biological state can be evaluated by conjugating genes encoding these proteins or proteins functionally equivalent to the individual proteins to an expression vector to introduce the genes into cells. The individual genes may be introduced in one vector or different vectors. As the RXR

10   gene, there may be used for example human RXRα gene (GenBank Accession No. NM_002957). As the co-activator, for example, human CBP gene (GenBank Accession No. U47741) and human SRC-1 gene (GenBank Accession No. U40396) may be used.

As the method for introducing the DNA construct

15   carrying the PPAR reporter gene and the PPARδ gene, transformation method by any of general calcium phosphate method, liposome method, lipofectin method and electroporation method may satisfactorily be used with no specific limitation. More preferably, electroporation method

20   is used.

Among compounds with PPARδ agonist activity as selected by the methods, a compound with preferable properties such as great neuroprotective action can be reselected. Specifically, a PPARδ agonist-derived test

25   substance is added together with a certain neurotoxic substance to a cell for culturing, to count viable cells and

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compare the count with a cell count when only the neurotoxic substance is added to the cell, as carried out in a series of experiments (Example 1 through Example 6) made by the inventors. Through interactions of test substances, a test

5   substance significantly suppressing the death of nerve cell as caused by a compound inducing certain neurotoxicity in a cell derived from the nerve system to reduce the lethal influence and increase the survival rate of the cell is then selected.

10   The entirety of the molecular mechanism of the death of nerve cell due to cerebral infarction has not yet been fully identified. By using for example thapsigargin as a substance inducing neurotoxicity, a person with ordinary experimental skills in the art can readily construct an assay

15   system in a mimetic manner, using a culture cell for selecting a more optimized compound as an active ingredient of a therapeutic agent for cerebral infarction and the like. Thapsigargin is known as a potent inhibitor against calcium pump in sarcoplasmic reticulum (SR) and endoplasmic

20   reticulum. Calcium is widely used for controlling cellular response, while calcium pump plays a very important role in maintaining cellular homeostasis. A report suggests that in the ischemic state at the acute stage of cerebral infarction, the disorder of intracellular calcium concentration in the

25   neuron in the vicinity of cerebral infarction is one cause of cellular death.

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It is considered that the concentration of thapsigargin for use in the assay system is a concentration of 1 nM or more to 1 µM or less, more preferably about 100 nM. The timing of adding thapsigargin is satisfactorily

5   before or after the addition of test compounds. More preferably, thapsigargin is added 2 hours after the addition of test compounds. The activity of test compounds for protecting nerve cells in the assay system can be determined by any of the following methods, after overnight culturing in

10   the concurrent presence of thapsigargin and test compounds. Any of the methods can readily be carried out by using any of commercially available kit products by a person with ordinary experimental skills in the art.

(1)   Assaying viable cells by MTT assay.

15   **(2)**   Assaying dead cells by LDH assay.

(3)   Detecting apoptosis by caspase-3/7 assay.

When a certain test compound is added together with thapsigargin to cells in the assay system for culturing and when the significant increase of the survival rate of the

20   cell is observed compared with the case of adding thapsigargin alone, the test compound possibly is the active component of a pharmaceutical agent effective for the therapeutic treatment of neurodegenerative diseases such as cerebral infarction.

25   As the culture cell for use in the assay system, a culture cell retaining the properties of human nerve cell is

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preferable. Cells of human neuroblastoma-derived established cell lines are preferably used. SH-SY5Y cell is more preferably used. It is considered that the use of the cell allows the establishment of an *in vitro* assay system of

5   cellular death, which is similar to the mechanism of the death of nerve cell. Actually, both the compounds L-165041 and GW501516 with effects on the suppression of cellular death in the assay system significantly exert the effect on the suppression of cellular death in cerebral infarction

10   models *in vivo*. Accordingly, a very good relation between the *in vitro* and *in vivo* effects is verified.

     The entirety of the molecular mechanism of the death of cellular cell due to Parkinson's disease has not yet been

15   fully elucidated. By using for example MPP$^+$ (1-methyl-4-phenylpyridinium ion) as a substance triggering neurotoxicity, a cell system using a culture cell so as to select an optimized compound as an active ingredient in a therapeutic agent for Parkinson's disease can be constructed

20   in a mimetic manner. One neurotoxin 1-methyl-4-phenyl-1,2,3,6tetrahydropyridine (MPTP) is known to induce Parkinsonism in humans and other primates and has drawn attention from the standpoint of the relation with the onset mechanism of Parkinson's disease. It is understood that

25   after MPTP is incorporated in brain, MPTP is metabolized into MPP$^+$ (1-methyl-4-phenylpyridinium ion) with monoamine oxidase

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B (MAOB) in astrocytes. MPP^+ is incorporated into dopamine neurons with a dopamine transporter existing in the cell membrane of dopaminergic neurons. Further, the MPP^+ incorporated in the dopamine neurons strongly inhibits the

5   complex I in the electron transmission system in mitochondria, to exert its cellular toxicity and thereby induce symptoms similar to Parkinsonism. Because of such reasons, currently, endogenous or exogenous MPTP-analogous substances are suspected as substances causing Parkinson's

10   disease. Currently, various research works are under way in various fields to verify whether or not MPTP-analogous substances selectively inhibiting dopaminergic neurons are contained in foods. Additionally, the possibility of a pharmaceutical agent reducing the neurotoxicity of MPP^+ as a

15   therapeutic agent for Parkinson's disease is promising.

It is considered that the concentration of MPP^+ for use in the assay system is a concentration of 100 nM or more to 10 mM or less, preferably about 3 mM. The timing of adding MPP^+ is satisfactorily before or after the addition of

20   test compounds. More preferably, MPP^+ is added 2 hours after the addition of test compounds. The activity of test compounds for protecting nerve cells in the assay system can be determined by any of the following methods, after overnight culturing in the concurrent presence of MPP^+ and

25   test compounds. Any of the methods can readily be carried

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out by using any of commercially available kit products, by a person with ordinary experimental skills in the art.

(1)   Assaying viable cells by MTT assay.

(2)   Assaying dead cells by LDH assay.

5   (3)   Detecting apoptosis by caspase-3/7 assay.

When a certain test compound is added together with MPP$^+$ to cells in the assay system for culturing and when the significant increase of the survival rate of the cell is observed, compared with the case of adding MPP$^+$ alone the

10   test compound is possibly the active component of a pharmaceutical agent effective for the therapeutic treatment of Parkinson's disease.

As the culture cell for use in the assay system, a culture cell retaining the properties of the dopaminergic

15   nerve cell in human mesencephalic nigra is ideally preferable. Because a considerable part of the pathway causing cellular death is possibly due to the mechanism common to overall cells of the nerve system, cells of relatively readily handleable human neuroblastoma-derived

20   established cell lines are now used instead. Cells of any human neuroblastoma-derived established cell line may be used. More preferably, SH-SY5Y cell can be used. It is considered that the use of the cell allows the establishment of an *in vitro* assay system of cellular death, which is

25   similar to the mechanism of the death of nerve cell due to Parkinson's disease. Actually, both the compounds L-165041

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and GW501516 with effects on the suppression of cellular

death in the assay system significantly exert effects on the

suppression of cellular death in an *in vivo* model of

Parkinson's disease. Therefore, a very good correlation

5   between the *in vitro* and *in vivo* effects is verified.

By using for example staurosporine instead of

thapsigargin and MPP^+ as a substance inducing neurotoxicity,

a person with ordinary experimental skills in the art can

readily construct an assay system using a culture cell for

10   selecting an optimized compound as an active ingredient of a

therapeutic agent for various neurodegenerative diseases in a

mimetic manner. Staurosporine is one of microbial alkaloids

generated by *Streptomyces staurosporeus* and is known as a

non-specific inhibitor interacting with highly homologous

15   catalyst regions among various numerous protein kinases.

Although the entirety of the detailed molecular mechanism and

the relation thereof with the diseases have not yet been

fully elucidated, it is known that staurosporine is capable

of widely inhibiting protein kinases having important

20   functions in intracellular information transduction, leading

to the death of nerve cells. For example, the disorder of

the cellular information transduction pathway in the neuron

in the vicinity of cerebral infarction lesion at the ischemic

state at the acute stage of cerebral infarction is suggested

25   as one cause triggering cellular death. Such disorder of

intracellular information transduction may be converged into

27

a mechanism common to numerous neurodegenerative diseases, which will lead to cellular death.

It is considered that the concentration of staurosporine for use in the assay system is a concentration of 1 nM or more to 1 μM or less, preferably about 150 nM. The timing of adding staurosporine is satisfactorily before or after the addition of test compounds. More preferably, staurosporine is added 2 hours after the addition of test compounds. The activity of test compounds for protecting nerve cells in the assay system can be determined by any of the following methods, after overnight culturing in the concurrent presence of staurosporine and test compounds. Any of the methods can readily be carried out by using any of commercially available kit products by a person with ordinary experimental skills in the art.

(1)   Assaying viable cells by MTT assay.

(2)   Assaying dead cells by LDH assay.

(3)   Detecting apoptosis by caspase-3/7 assay.

When a certain test compound is added together with staurosporine to cells in the assay system for culturing, the test compound is possibly the active component of a pharmaceutical agent effective for the therapeutic treatment of neurodegenerative diseases such as cerebral infarction when the significant increase of the survival rate of the cell is observed, compared with the case of adding staurosporine alone. As the culture cell for use in the

assay system, a culture cell retaining the properties of
human nerve cell is preferable. Cells of human
neuroblastoma-derived established cell lines are preferably
used. SH-SY5Y cell is more preferably used. It is

5   considered that the use of the cell allows the establishment
of an *in vitro* assay system of cellular death, which is
similar to the mechanism of the death of nerve cell.
Actually, both the compounds L-165041 and GW501516 with
actual effects on the suppression of cellular death in the

10   assay system significantly exert the effect on the
suppression of cellular death even in cerebral infarction
models *in vivo*. Accordingly, a very good correlation between
the *in vitro* and *in vivo* effects is verified.

The "PPARδ agonist specifically reselected using the

15   activity of suppressing cellular death as a marker" in
accordance with the invention is a compound obtained by
reselecting a compound with a greater activity of suppressing
cellular death by the method using a culture cell with
thapsigargin, MPP^+ or staurosporine added, from PPARδ

20   agonists selected by known methods such as the reporter gene
assay method. As the intensity of the activity of
suppressing cellular death, the viable cell count assayed by
the MTT assay method with the compound added at an optimal
concentration within the range of 0.1 to 100 μM under the

25   conditions described above is improved at least at 10 %,

preferably at 30 % or more and more preferably at 50 % or more, compared with the case of never adding such compound.

The invention relates to a therapeutic method for Alzheimer's disease, Parkinson's disease, cerebral infarction, head injuries, cerebral hemorrhage, spinal injuries, multiple sclerosis, amyotrophic lateral sclerosis, Huntington's disease, diabetic or drug-induced peripheral nerve disorders or retinal nerve disorders, as well as a therapeutic agent thereof containing a PPARδ agonist as an active ingredient.

The compound obtained by selecting a PPARδ agonist on the basis of the assessment method described above is useful for the therapeutic treatment and prophylaxis of the following diseases probably occurring via the degeneration of nerve cells.

Alzheimer's disease, Parkinson's disease, cerebral infarction, head injuries, cerebral hemorrhage, spinal injuries, multiple sclerosis, amyotrophic lateral sclerosis, Huntington's disease, diabetic or drug-induced peripheral nerve disorders or retinal nerve disorders.

For using the PPARδ agonist in accordance with the invention as a pharmaceutical agent, the PPARδ agonist itself can be used as such. However, the PPARδ agonist may be formulated and used by known pharmaceutical methods. The therapeutic agent may be provided in any of forms including oral agent, parenteral agent or external agent. An

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administration route appropriate for the subject disease to be treated and an optimal dosage form for a subject person to be administered can be selected. For example, injections, infusions, syrups, tablets, granules, powders, troches,

5 pills, pellets, capsules, microcapsules, suppositories, creams, ointments, aerosols, inhalation powders, liquids, emulsions, suspensions, enteric coating agents, sprays, eye drops, nasal drops and other appropriate dosage forms suitable for use may be possible and can be mixed with

10 pharmaceutically acceptable, routine non-toxic carriers. If necessary, additionally, auxiliary agents, stabilizers, thickeners, coloring agents and flavor may be used. Such pharmaceutical formulations may be produced by routine methods using organic or inorganic various carriers common

15 for formulation, including for example excipients (for example, sucrose, starch, mannit, sorbit, lactose, glucose, cellulose, talc, calcium phosphate, and calcium carbonate), binders (for example, cellulose, methyl cellulose, hydroxymethyl cellulose, polypropylpyrrolidone, gelatin, gum

20 arabic, polyethylene glycol, sucrose and starch), disintegrators (for example, starch, carboxymethyl cellulose, hydroxypropyl starch, sodium hydrogen carbonate, calcium phosphate, and calcium citrate), lubricants (for example, magnesium stearate, aerosol, talc, and sodium lauryl

25 sulfate), flavor (for example, citric acid, menthol, glycine and orange powder), preservatives (for example, sodium

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benzoate, sodium bisulfite, methyl paraben, and propyl

paraben), stabilizers (for example, citric acid, sodium

citrate and acetic acid), suspending agents (for example,

methyl cellulose, polyvinylpyrrolidone, and aluminium

5   stearate), dispersants (for example, hydroxypropylmethyl

cellulose), diluents (for example, water), base waxes (for

example, cacao butter, white vaseline, and polyethylene

glycol).

For producing these formulations, the pharmaceutical

10   composition may satisfactorily contain pharmaceutically

acceptable salts at an amount sufficient enough for the

desired pharmaceutical effect to be exerted for the course or

state of the disease. These salts may be used in the forms

of pharmaceutical formulations in solid, semi-solid or

15   liquid. Such pharmaceutically acceptable salts include

routine non-toxic salts and specifically include for example

metal salts such as alkali metal salts (for example, sodium

salts or potassium salts) and alkali earth metal salts (for

example, calcium salts or magnesium salts), inorganic acid-

20   added salts (for example, hydrochloride salts, hydrobromide

salts, sulfate salts and phosphate salts), organic carboxylic

acid or sulfonic acid-added salts (for example, formate

salts, acetate salts, trifluoroacetate salts, maleate salts,

tartrate salts, fumarate salts, methanesulfonate salts,

25   benzenesulfonate salts, and toluenesulfonate salts), and

salts with basic or acidic amino acids (for example, arginine, aspartic acid and glutamic acid).

For administering the formulations to patients, these formulations are suitable for administration into nose and

5   eye, external administration (topical), administration into rectum and lung (injections through nose or mouse), oral or parenteral (intraventricular, subcutaneous, intravenous and intramuscular) administration or inhalation. Administration via injections can be done by known methods such as

10 intraarterial injections, intravenous injections and subcutaneous injections.

The dose of the therapeutic agent for the invention is an amount enough for the desired therapeutic effect to be exerted. The therapeutically effective amount of the

15 compound is generally about 0.1 to 100 mg per day, preferably 1 to 16 mg per day for parenteral administration. The effective single dose is selected within a range of 0.001 to 1 mg per 1 kg • patient body weight, preferably 0.01 to 0.16 mg per 1 kg • patient body weight. Nonetheless, the dose may

20 be modified in a manner depending on the body weight, age and symptoms of each patient to be treated and the dosing method and the like to be employed. A person with ordinary experimental skills in the art can appropriately select a more suitable dose on the basis of data from animal

25 experiments and the like.

Brief Description of the Drawings

Best Mode for Carrying out the Invention

The invention is now described in detail in the following Examples. However, the invention is never limited to the Examples. The references in the related art as cited in the specification are all incorporated by reference in the specification.

Example 1

(1)   Action of PPAR-• agonist on the suppression of cellular death in thapsigargin-induced cellular death model (neurodegenerative disease model)

<Method>

Assaying viable cells by MTT assay

SH-SY5Y cells were plated on a 96-well plate (70,000 cell/well in 100 µl DMEM low glucose 10 % fetal bovine serum) for overnight culturing, from which the culture medium was removed with an aspirator. Then, DMEM without serum was added at 50 µl/well. DMEM without serum containing a pharmaceutical agent (L-165041 or GW501516) at a concentration 2 × was added at 50 µl/well (for a blank, DMEM without serum alone was added). 2 hours later, DMEM without serum containing 600 nM thapsigargin was added at 20 µl/well. (Thapsigargin to a final 100 nM concentration) (For a

control, DMEM without serum alone was added.) 24 hours

later, the viable cell count was calculated from the

absorbance at 490 nm, using Celltiter 96 Aqueous one solution

cell proliferation assay kit (Promega).

5   Table 1 shows the relation between the various

concentrations of L-165041 or GW501516 added and the viable

cell count thus determined. The results show the effect of

the PPARδ agonist on the suppression of thapsigargin-induced

cellular death.

10

Table 1

	(•M)	Thapsigargin	MTT assay A490
L-165041	0	−	0.5536 ± 0.0060
	0	+	0.2156 ± 0.0043
	0.1	+	0.2176 ± 0.0036
	1	+	0.2158 ± 0.0034
	10	+	0.2436 ± 0.0015**
	100	+	0.4405 ± 0.0065**
GW501516	0	−	0.7658 ± 0.0048
	0	+	0.2823 ± 0.0070
	0.1	+	0.2978 ± 0.0096
	1	+	0.3150 ± 0.0135
	10	+	0.3478 ± 0.0110**
	100	+	0.2950 ± 0.0033**

Mean ± standard deviation (n = 4)

** $P < 0.01$ (one-way ANOVA followed by Dunnett's test vs

thapsigargin-only treatment)

15

        The individual assay values at A_{490} in Table 1 are

expressed in mean ± standard deviation from independent 4

experiments (n = 4). Among the control group with no treatment with any PPARδ agonist, the two groups of the thapsigargin-treated group and no thapsigargin-treated group were comparatively tested by Student's t-test. Significance

5   at $p < 0.01$ was verified. For comparatively testing the individual groups treated with thapsigargin and various concentrations of the PPARδ agonist (L-165041 or GW501516), the difference in variance among the individual groups was verified by one-way ANOVA and subsequently tested by

10 Dunnett's test. Significance at $p < 0.01$ is marked with asterisk (**) in Table 1.

In the assay system, cellular death is induced by adding thapsigargin (TG) as an inhibitor of CA^{2+} ATPase in

15 endoplasmic reticulum to a neuroblastoma SH-SY5Y to give stresses to endoplasmic reticulum. Cellular death due to the stresses to endoplasmic reticulum is considered as a cause of the death of nerve cells at ischemic state. Accordingly, the assay system can be said as an assay system mimicking the

20 death of nerve cells at ischemic state. The experiments indicated that L-165041 and GW501516 suppressed cellular death induced by thapsigargin (TG) in a concentration-dependent manner. (The cellular toxicity of GW501516 can be observed when GW501516 is at a concentration of 100 μM.)

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Example 2

(2)   Action of PPAR-• agonist on the suppression of
cellular death in thapsigargin-induced cellular death model
(neurodegenerative disease model)

5   <Method>

Assaying dead cells by LDH assay

        SH-SY5Y cells were plated on a 96-well plate (70,000
cell/well in 100 μl DMEM low glucose 10 % fetal bovine serum)
for overnight culturing, from which the culture medium was
10   removed with an aspirator. Then, DMEM without serum was
added at 50 μl/well. DMEM without serum containing a
pharmaceutical agent (L-165041 or GW501516) at a
concentration 2 × was added at 50 μl/well (for a blank, DMEM
without serum alone was added). 2 hours later, DMEM without
15   serum containing 600 nM thapsigargin was added at 20 μl/well.
(Thapsigargin to a final 100 nM concentration) (For a
control, DMEM without serum alone was added.) 24 hours
later, the absorbance at 490 nm was assayed with a
cytotoxicity detection kit (Roche) to assay the LDH activity.
20   Table 2 shows the assay results of dead cells thus determined
by LDH assay. An apparent correlation between the result of
the viable cell count as determined by MTT assay in Example 1
and the LDH activity is shown. The above results clearly
indicate that the PPARδ agonists are effective on the
25   suppression of the thapsigargin-induced cellular death.

Table 2

		Thapsigargin	LDH release A490
L-165041		-	0.2258 ± 0.0049
		+	0.6750 ± 0.0106
		+	0.6758 ± 0.0183
		+	0.6295 ± 0.0139
		+	0.6029 ± 0.0135**
		+	0.2987 ± 0.0128**
GW501516		-	0.1088 ± 0.0060
		+	0.5413 ± 0.0171
		+	0.6225 ± 0.0229
		+	0.5750 ± 0.0212
		+	0.3325 ± 0.0259**
		+	0.6675 ± 0.0328**

Mean ± standard deviation (n = 4)

** $P < 0.01$ (one-way ANOVA followed by Dunnett's test vs thapsigargin-only treatment)

Example 3

(3)   Action of PPAR-• agonist on the suppression of cellular death in thapsigargin-induced cellular death model (neurodegenerative disease model)

<Method>

Detecting apoptosis by caspase-3/7 assay

SH-SY5Y cells were spread on a 96-well plate (70,000 cell/well in 100 μl DMEM low glucose 10 % fetal bovine serum) for overnight culturing, from which the culture medium was removed with an aspirator. Then, DMEM without serum was added at 50 μl/well. DMEM without serum containing a pharmaceutical agent (L-165041 or GW501516) at a

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concentration 2 × was added at 50 μl/well (for a blank, DMEM without serum alone was added). 2 hours later, DMEM without serum containing 600 nM thapsigargin was added at 20 μl/well well. (Thapsigargin to a final 100 nM concentration) (For a control, DMEM without serum alone was added.)

3 hours later, the caspase-3/7 activity was assayed with Apo-One homogenous Caspase-3/7 assay kit (Promega). Table 3 shows the results.

Since the PPARδ agonists L-165041 and GW501516 suppress the activity of caspase-3/7 activated during apoptosis induction, apparently, both these compounds directly or indirectly suppress apoptosis signal, so that these compounds are effective on the suppression of thapsigargin-induced cellular death.

Table 3

	(μM)	thapsigargin	Caspase 3/7 activity RFLU
L-165041	0	−	147789 ± 5193
	0	+	702332 ± 14470
	0.1	+	732549 ± 24888
	1	+	652843 ± 8229
	10	+	554233 ± 23703**
	100	+	242770 ± 5414**
GW501516	0	−	159016 ± 2356
	0	+	276057 ± 11115
	0.1	+	226861 ± 8501*
	1	+	237165 ± 5248
	10	+	125951 ± 6465**
	100	+	421972 ± 21782

Mean ± standard deviation (n = 4)

* p < 0.05

** P < 0.01 (one-way ANOVA followed by Dunnett's test vs thapsigargin-only treatment)

Example 4

(1)   Action of PPAR-• agonist on the suppression of cellular death in MPP$^+$-induced cellular death model (Parkinson's disease model)

<Method>

Assaying viable cells by MTT assay

SH-SY5Y cells were spread on a 96-well plate (70,000 cell/well in 100 μl DMEM low glucose 10 % fetal bovine serum) for overnight culturing, from which the culture medium was removed with an aspirator. Then, DMEM without serum was added at 50 μl/well. DMEM without serum containing a

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pharmaceutical agent (L-165041 or GW501516) at a concentration 2 × was added at 50 μl/well (for a blank, DMEM without serum alone was added). 2 hours later, DMEM without serum containing 18 mM MPP$^+$ was added at 20 μl/well. (MPP$^+$ to

5   a final 3 mM concentration) (For a control, DMEM without serum alone was added).

24 hours later, cell growth activity was assayed by measuring the absorbance at 490 nm, using Celltiter 96 aqueous one solution cell proliferation assay kit (Promega).

10   Table 4 shows the results. The results apparently show that the PPARδ agonists are effective on the suppression of MPP$^+$-induced cellular death.

In the assay system, MPP$^+$ as an MPTP metabolite was added to a neuroblastoma SH-SY5Y, to inhibit the

15   mitochondrial complex I to cause the generation of active oxygen and the inhibition of ATP synthesis, to induce cellular death. It is said that in the murine MPTP model commonly used as a model of Parkinson's disease, MPTP passing through the blood brain barrier is metabolized into MPP$^+$,

20   which exerts toxicity specific to murine nigra cell to induce nerve detachment. Therefore, the assay system can be said as an *in vitro* assay system mimicking the murine MPTP model. The experiments verify that L-165041 and GW501516 suppress cellular death induced by MPP$^+$ in a concentration-dependent

25   manner.

Table 4

	(µM)	MPP$^+$	MTT assay A490
L-165041	0	−	0.5448 ± 0.0041
	0	+	0.3392 ± 0.0048
	0.1	+	0.3744 ± 0.0067*
	1	+	0.3935 ± 0.0083**
	10	+	0.4799 ± 0.0078**
	100	+	0.3181 ± 0.0122
GW501516	0	−	0.7447 ± 0.0054
	0	+	0.3178 ± 0.0031
	0.1	+	0.3361 ± 0.0073
	1	+	0.3926 ± 0.0090**
	10	+	0.3814 ± 0.0156**
	100	+	0.2990 ± 0.0144

Mean ± standard deviation (n = 4)

* P < 0.05

** p < 0.01 (one-way ANOVA followed by Dunnett's test vs

5 MPP$^+$-only treatment)

Example 5

(2)   Action of PPAR-• agonist on the suppression of

cellular death in MPP$^+$-induced cellular death model

10 (Parkinson's disease model)

<Method>

Assaying dead cells by LDH assay

SH-SY5Y cells were spread on a 96-well plate (70,000

cell/well in 100 µl DMEM low glucose 10 % fetal bovine serum)

15 for overnight culturing, from which the culture medium was

removed with an aspirator. Then, DMEM without serum was

added at 50 µl/well. DMEM without serum containing a

pharmaceutical agent (L-165041 or GW501516) at a

concentration 2 × was added at 50 μl/well (for a blank, DMEM

without serum alone was added). 2 hours later, DMEM without

serum containing 18 mM MPP$^+$ was added at 20 μl/well. (MPP$^+$ to

5   a final 3 mM concentration) (For a control, DMEM without

serum alone was added.)

       24 hours later, the dead cell count was calculated on

the basis of the LDH activity assayed with a cytotoxicity

detection kit (Roche). Table 5 shows the results. The

10   results reveal that the PPARδ agonists are effective on the

suppression of MPP$^+$-induced cellular death. It was verified

by the correlation between the assay results of cells in

Example 4 and the results of dead cells by LDH assay from

this experiment that L-165041 and GW501516 suppressed

15   cellular death induced by MPP$^+$ in a concentration-dependent

manner. (Under the present assay conditions, it was observed

that both L-165041 and GW501516 exerted cytotoxicity at the

concentration of 100 μM.)

Table 5

	(µM)	MPP$^+$	LDH release A490
L-165041	0	−	0.2807 ± 0.0058
	0	+	0.4617 ± 0.0081
	0.1	+	0.3980 ± 0.0141*
	1	+	0.3874 ± 0.0120**
	10	+	0.3133 ± 0.0024**
	100	+	0.6709 ± 0.0093
GW501516	0	−	0.1610 ± 0.0052
	0	+	0.3772 ± 0.0187
	0.1	+	0.3633 ± 0.0153
	1	+	0.3222 ± 0.0207*
	10	+	0.2981 ± 0.0154**
	100	+	0.7969 ± 0.0251

Mean ± standard deviation (n = 4)

* P < 0.05

** p < 0.01 (one-way ANOVA followed by Dunnett's test vs
MPP$^+$-only treatment)

Example 6

(3)   Action of PPAR-• agonist on the suppression of
cellular death in MPP$^+$-induced cellular death model
(Parkinson's disease model)

<Method>

Detecting apoptosis by caspase-3/7 assay

        SH-SY5Y cells were spread on a 96-well plate (70,000
cell/well in 100 µl DMEM low glucose 10 % fetal bovine serum)
for overnight culturing, from which the culture medium was
removed with an aspirator. Then, DMEM without serum was
added at 50 µl/well. DMEM without serum containing a

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pharmaceutical agent (L-165041 or GW501516) at a concentration 2 × was added at 50 μl/well (for a blank, DMEM without serum alone was added). 2 hours later, DMEM without serum containing 18 mM MPP$^+$ was added at 20 μl/well. (MPP$^+$ to

5   a final 3 mM concentration.) (For control, DMEM without serum alone was added.)

3 hours later, the caspase-3/7 activity was assayed with Apo-One homogenous Caspase-3/7 assay kit (Promega). Table 6 shows the results.

10   Since the PPARδ agonists L-165041 and GW501516 both suppress the activity of caspase-3/7 in a concentration-dependent manner, the results indicate that both these compounds directly or indirectly suppress apoptosis signal, so that these compounds exert an effect on the suppression of

15   MPP$^+$-induced cellular death.

45

Table 6

	(μM)	MPP$^+$	Caspase 3/7 activity RFLU
L-165041	0	−	185992 ± 3185
	0	+	1455497 ± 28684
	0.1	+	1430206 ± 30804
	1	+	1168920 ± 30554**
	10	+	646736 ± 26366**
	100	+	572152 ± 33695**
GW501516	0	−	114533 ± 5938
	0	+	933722 ± 18553
	0.1	+	847552 ± 36575
	1	+	621059 ± 26590**
	10	+	798646 ± 54365
	100	+	398604 ± 55698**

Mean ± standard deviation (n = 4)

* P < 0.05

** p < 0.01 (one-way ANOVA followed by Dunnett's test vs

5   MPP$^+$-only treatment)

Reference Example 1

Action profiles and PPAR selectivities of L-165041 and

GW501516 by *in vitro* receptor gene assay

10   Human PPARα cDNA, human PPARδ cDNA, human PPARγ cDNA,

murine PPARα cDNA, murine PPARδ cDNA and murine PPARγ cDNA

were individually introduced in the multi-cloning site of an

expression vector pBIND (manufactured by Promega), to

construct GAL4-human PPARα fusion protein-expressing vector

15   pBINDhPPARα, GAL4-human PPARδ fusion protein-expressing

vector pBINDhPPARδ, GAL4-human PPARγ fusion protein-

expressing vector pBINDhPPARγ, GAL4-murine PPARα fusion

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protein-expressing vector pBINDmPPARα, GAL4-murine PPARδ

fusion protein-expressing vector pBINDmPPARδ, and GAL4-murine

PPARγ fusion protein-expressing vector pBINDmPPARγ. Together

with a vector pG5luc (manufactured by Promega) for reporter

5 gene expression, these expression vectors were individually

introduced into African green monkey kidney-derived CV-1 cell

using LipofectAMINE Reagent (manufactured by GIBCO BRL), to

which a test compound L-165041 or GW501516 was added, for

overnight culturing under conditions of 37°C and saturated

10 humidity in the presence of 5 % CO_2. Then, firefly

luciferase activity and Renilla luciferase activity were

determined, using Dual-Luciferase Reporter Assay System

(manufactured by Promega). The action of each PPAR sub-type

to activate transcription was determined on the basis of the

15 activity of firefly luciferase as a pG5luc reporter, using

the activity of Renilla luciferase derived from pBIND as an

internal standard. Table 7 shows the action profiles and

PPAR selectivities of L-165041 and GW501516 by the *in vitro*

receptor gene assay.

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Table 7

	Concentration (•M) of L-165041 for action exertion	Concentration (•M) of GW501516 for action exertion
Human PPAR-•	10	1
Human PPAR-•	10	>10
Human PPAR-•	0.1	<0.01
Murine PPAR-•	>100	10
Murine PPAR-•	10	>10
Murine PPAR-•	0.1	0.01

Example 7

Action of PPAR-• agonist action in rat cerebral infarction

5   model

Various compounds were evaluated in a rat cerebral infarction model. It was verified that L-165041 (PPAR-δ agonist) and GW501516 (PPAR-δ agonist) had potent actions to reduce cerebral infarction.

10   <Method>

Assessment of PPAR-δ agonist in cerebral infarction model (according to the Koizumi method)

L-165041 and GW501516 were dissolved in polyethylene glycol (PEG300). The pharmaceutical agents were given to

15   male Wistar rats (age of 9 weeks), using an ALZET osmotic pressure mini-pump preliminarily filled aseptically with the pharmaceutical agents. On the day before a surgery for cerebral ischemia, a guide cannula was inserted in the right ventricule, namely at 0.8 mm backward from the coronal suture

20   of the skull, and 1.5 mm unilaterally on the right side and

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in a 4.0-mm depth. Then, ventricular infusion was started at

a flow rate of 1 μL/hour. The solvent was given to a control

group in the same way. The infusion was sustained until the

rats were sacrificed to death. As a cerebral infarction

5   model, a middle cerebral artery-occluded reperfusion model

according to the Koizumi's method was used. Specifically, a

4-0 nylon embolus with silicon coating and of a 19-mm length

was inserted from the branch part of right common carotid

artery toward internal carotid artery in the rats under

10   anesthesia with halothane (initially at 4 % and retained at

1.5 %), to occlude the right middle cerebral artery.   90

minutes after ischemia, the nylon embolus was taken out under

anesthesia again, for reperfusion. 24 hours after the

reperfusion of cerebral ischemia, brain was resected, to

15   prepare continuous coronary sections of a 2-mm thickness.

The sections were stained with 2 % TTC (triphenyltetrazolium

chloride) solution, to measure the damaged area of the brain

and calculate the brain damage ratio.

Table 8 shows the results of measured cerebral damaged

20   areas thus obtained. Table 9 shows the results of the

assessment of the action of reducing cerebral infarction.

The results in Table 9 show that both L-165041 and

GW501516 both have actions of reducing cerebral damages in a

dose-dependent manner in the cerebral infarction model

25   (according to the Koizumi's method). Further, GW501516 had a

far greater action of reducing cerebral damages. The

difference in the intensity of the action of reducing

cerebral infarction between the two agents correlates well

with the difference in the intensity of the action between

the human and murine PPARδ agonists as measured by the *in*

5 *vitro* reporter gene assay (Table 7). These results suggest

that PPARδ agonists generally have an action of reducing

cerebral infarction.

Table 8

10

Action of PPAR-δ agonists in rat cerebral infarction model
Lesions with cerebral infarction (area in % of damaged site
in the 6 sections)

	Control group	L-165041		GW501516	
μg/head/day		24	240	24	240
Number of animals	(n = 8)	(n = 9)	(n = 9)	(n = 9)	(n = 9)
Total	23.68 ± 1.96	22.68 ± 0.83	19.40 ± 1.98	18.74 ± 2.00	15.14 ± 2.20*
Cortex	13.93 ± 1.23	12.70 ± 0.63	10.22 ± 1.29*	9.39 ± 1.56	7.26 ± 1.67*
Subcortex	9.74 ± 0.82	9.98 ± 0.30	9.19 ± 0.85	9.35 ± 0.65	7.88 ± 0.79

Mean ± standard deviation

15 * $P < 0.05$ (one way ANOVA followed by Dunnett's test vs

control group)

Table 9

Action of reducing cerebral infarction

	L-165041		GW501516	
i.c.v.(•g/ head/day)	24	240	24	240
Reduction ratio of cerebral cortex damage	9 %	27 %	33 %	48 %

Example 8

5 Action of PPAR-δ agonists in murine MPTP Parkinson's model

For the purpose of examining the neuroprotective

action of PPAR• δ agonists *in vivo*, murine MPTP Parkinson's

model was used.

<Method>

10 Assessment of PPAR-δ agonist in murine MPTP Parkinson's model

After male 9-week-old C57BL/6 mice non-fasted were

anesthetized with pentobarbital (60 mg/kg, i.p.), the mice

were fixed on a cerebral stereotactic apparatus, for fixing L

cannula on the skull bone (at 0.0 mm to bregma, 1.2 mm

15 lateral to midline, 2.5 mm ventral from skull) and

subsequently embedding an ALZET osmotic pressure pump

subcutaneously on a dorsal part. PPAR-δ agonists (L-165041

and GW501516) were dissolved in 30 % DMSO/saline, for

filtration and sterilization. Subsequently, the

20 pharmaceutical agents were aseptically charged in the osmotic

pressure pump and then infused into the ventricule at 12 μg

per day or 120 μg per day at a flow rate of 0.5 μL/hr. 2

days after the embedding of the pump, MPTP (20 mg/kg) was

intraperitoneally administered twice at a 2-hour interval. 4
days after MPTP administration, the striate body was resected
to assay the contents of dopamine (DA) and its metabolites
3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid

5   (HVA) in the striate body by HPLC-ECD (high-performance
liquid chromatography with electrochemical detection).

Table 10 shows the contents of DA and its metabolites
per wet weight of striate body in the MPTP Parkinson's
disease model as assayed in such manner. Additionally, Table

10   11 shows the resumption ratio of the contents of DA and its
metabolites as calculated from the results in Table 10.
These results indicate that the PPARδ agonists have an action
of suppressing the decrease of the contents of DA and its
metabolites DOPAC and HVA in the striate body due to MPTP in

15   the murine MPTP Parkinson's disease model. The action of
suppressing the decrease of the contents of DA and its
metabolites DOPAC and HVA was greatly observed with L-165041
at a dose of 120 μg/head/day but not so greatly observed at a
dose of 12 μg/head/day. The action was greatly observed with

20   GW501516 at any of the doses. The difference in the
intensity of the action of suppressing the decrease of DA
content between these two agents correlates very well with
the difference in the intensity of the agonist action between
the human and murine PPARδ agonists as assayed by the *in*

25   *vitro* reporter gene assay (Table 7). These results indicate
that the actions of these compounds for suppressing the

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decrease of the contents of DA and its metabolites in striate body are generally ascribed to the PPAR-δ agonist activities.

Table 10

Contents of DA and its metabolites per wet weight of striate body in MPTP Parkinson's disease model

	Dose (μg/head /day)	MPTP treat ment	Contents		
			DA (ng/mg tissue)	DOPAC (ng/mg tissues)	HVA (ng/ mg tissue)
Normal	0	−	15.59 ± 0.38	0.76 ± 0.03	1.31 ± 0.02
Control	0	+	5.01 ± 0.39	0.29 ± 0.02	0.68 ± 0.03
GW501516	12	+	7.41 ± 1.27	0.39 ± 0.06	0.82 ± 0.08
	120	+	8.98 ± 1.20*	0.44 ± 0.04*	0.90 ± 0.05**
L-165041	12	+	5.06 ± 1.08	0.28 ± 0.05	0.66 ± 0.08
	120	+	7.81 ± 0.93*	0.44 ± 0.07	0.91 ± 0.10

Mean ± standard deviation

Normal group: n = 6; control group: n = 6; other individual groups: n = 7

* $p < 0.05$ (Student's t-test vs control)

** $p < 0.01$ (Student's t-test vs control)

Table 11

Resumption ratios of contents of DA and its metabolites in

MPTP Parkinson's disease model

	Dose (μg/head /day)	MPTP treat ment	Contents		
			DA (%)	DOPAC (%)	HVA (%)
Normal	0	−	100.00 ± 3.56	100.00 ± 6.18	100.00 ± 3.05
Control	0	+	0.00 ± 3.67	0.00 ± 4.44	0.00 ± 4.27
GW501516	12	+	22.67 ± 12.00	21.72 ± 11.73	21.42 ± 12.07
	120	+	37.53 ± 11.33*	32.44 ± 9.17*	33.93 ± 8.72**
L-165041	12	+	0.42 ± 10.20	-1.45 ± 10.89	-4.65 ± 12.88
	120	+	26.42 ± 8.83*	32.22 ± 15.41	36.58 ± 16.68

Mean ± standard deviation

* $p < 0.05$ (Student's t-test vs control)

** $p < 0.01$ (Student's t-test vs control)

Example 9

(1) Action of PPAR-• agonist on the suppression of cellular

death in staurosporine-induced cellular death model

Cellular death was induced by adding a protein kinase

inhibitor staurosporine to a neuroblastoma SH-SY5Y to examine

the effect of PPARδ agonists on the suppression of cellular

death.

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<Method>

Assaying viable cells by MTT assay

SH-SY5Y cells were plated on a 96-well plate (70,000 cell/well in 100 μl DMEM low glucose 10 % fetal bovine serum) for overnight culturing, from which the culture medium was removed with an aspirator. Then, DMEM without serum was added at 50 μl/well. DMEM without serum containing a pharmaceutical agent (L-165041 or GW501516) at a concentration 2 × was added at 50 μl/well (for a blank, DMEM without serum alone was added). 2 hours later, DMEM without serum containing 900 nM staurosporine was added at 20 μl/well. (Staurosporine to a final 150 nM concentration) (For a control, DMEM without serum alone was added.) 24 hours later, the viable cell count was calculated from the absorbance at 490 nm, using Celltiter 96 Aqueous one solution cell proliferation assay kit (Promega).

Table 1 shows the relation between the various concentrations of L-165041 or GW501516 added and the viable cell count thus determined. The results show the effects of PPARδ agonists (L-165041, GW501516) on the suppression of staurosporine-induced cellular death in a concentration-dependent manner.

Table 12

	(µM)	Staurosporine	MTT assay A490
L-165041	0	-	0.6607 ± 0.0022
	0	+	0.4249 ± 0.0086
	0.1	+	0.5523 ± 0.0209**
	1	+	0.5532 ± 0.0056**
	10	+	0.5167 ± 0.0098**
GW501516	0	-	0.6608 ± 0.0023
	0	+	0.4250 ± 0.0087
	0.1	+	0.5179 ± 0.0140**
	1	+	0.5367 ± 0.0149**
	10	+	0.5860 ± 0.0086**

Mean ± standard deviation (n = 4)

** $P < 0.01$ (one-way ANOVA followed by Dunnett's test vs staurosporine-alone treatment)

** $p < 0.01$ (Student's t-test vs control)

Example 11

(2)   Action of PPAR-• agonist on the suppression of cellular death in staurosporine-induced cellular death model

<Method>

Assaying dead cells by LDH assay

SH-SY5Y cells were plated on a 96-well plate (70,000 cell/well in 100 µl DMEM low glucose 10 % fetal bovine serum) for overnight culturing, from which the culture medium was removed with an aspirator. Then, DMEM without serum was added at 50 µl/well. DMEM without serum containing a pharmaceutical agent (L-165041 or GW501516) at a concentration 2 × was added at 50 µl/well (for a blank, DMEM

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without serum alone was added). 2 hours later, DMEM without

serum containing 900 nM staurosporine was added at 20

μl/well. (Staurosporine to a final 150 nM concentration) (For

a control, DMEM without serum alone was added.) 24 hours

5   later, LDH activity was assayed by measuring the absorbance

at 490 nm, using Cytotoxicity detection kit (Roche). Table 2

shows the assay results of dead cells by LDH assay as

determined in this manner. An apparent correlation is

observed with the results of viable cells as determined by

10 the MTT assay in Example 9. The results show that the PPARδ

agonists have an effect on the suppression of staurosporine-

induced cellular death.

Table 13

	(μM)	Staurosporine	LDH release A490
L-165041	0	−	0.1315 ± 0.0042
	0	+	0.6058 ± 0.0181
	0.1	+	0.5330 ± 0.0298
	1	+	0.5230 ± 0.0160
	10	+	0.5319 ± 0.0267
GW501516	0	−	0.1315 ± 0.0042
	0	+	0.6058 ± 0.0181
	0.1	+	0.5430 ± 0.0264
	1	+	0.5201 ± 0.0313
	10	+	0.4025 ± 0.0188**

15 Mean ± standard deviation (n = 4)

** $P < 0.01$ (one-way ANOVA followed by Dunnett's test vs

staurosporine-only treatment)

Example 11

(3)   Action of PPAR-● agonist on the suppression of
cellular death in staurosporine-induced cellular death model
<Method>

5 Detecting apoptosis by caspase-3/7 assay

SH-SY5Y cells were spread on a 96-well plate (70,000
cell/well in 100 μl DMEM low glucose 10 % fetal bovine serum)
for overnight culturing, from which the culture medium was
removed with an aspirator. Then, DMEM without serum was

10 added at 50 μl/well. DMEM without serum containing a
pharmaceutical agent (L-165041 or GW501516) at a
concentration 2 × was added at 50 μl/well (for a blank, DMEM
without serum alone was added). 2 hours later, DMEM without
serum containing 900 nM staurosporine was added at 20

15 μl/well. (Staurosporine to a final 150 nM concentration) (For
a control, DMEM without serum alone was added).

3 hours later, the caspase-3/7 activity was assayed
with Apo-One homogenous Caspase-3/7 assay kit (Promega).
Table 3 shows the results.

20 Since the PPARδ agonists L-165041 and GW501516
suppress the activity of caspase-3/7 activated during
apoptosis induction, apparently, both these compounds
directly or indirectly suppress apoptosis signal, so that
these compounds are effective on the suppression of

25 staurosporine-induced cellular death.

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Table 14

	(μM)	Staurosporine	Caspase 3/7 activity RFLU
L-165041	0	−	104281 ± 3078
	0	+	614433 ± 39923
	0.1	+	535404 ± 23301
	1	+	520732 ± 17273
	10	+	483017 ± 27259*
GW501516	0	−	104281 ± 3078
	0	+	614433 ± 39923
	0.1	+	586295 ± 18787
	1	+	463943 ± 12110**
	10	+	420844 ± 13962**

Mean ± standard deviation (n = 4)

* $p < 0.05$

** $P < 0.01$ (one-way ANOVA followed by Dunnett's test vs
staurosporine-only treatment)

Industrial Applicability

In accordance with the invention, a compound with a
protective action for nerve cell can be reselected by adding
PPARδ agonist to a culture cell system where toxic substances
such as thapsigargin, MPP^+ and staurosporine are
preliminarily allowed to react and reselecting a compound
improving the survival rate. The compound selected by such
method can be used as an active ingredient of a therapeutic
agent for neurodegenerative diseases such as cerebral
infarction and Parkinson's disease. Thus, the invention is
very useful for research works for creating novel
pharmaceutical agent.